Director's Semi-Annual Progress Report

No. 3

National Aeronautics

and

Space Administration

Research Grant NsG - 651

ORIGINAL
Grant Period: 21 months

Date begun: May 1, 1964

NASA Monitors: Dr. Freeman H. Quimby
and Dr. Cyril A. Ponnamperuma
Office of Life Sciences

Date: December 10, 1965

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I. Personnel

A. Dr. Frank Millich, Associate Professor
Department of Chemistry, U.M.K.C.....Project Director

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Department of Chemistry, U.M.K.C.....Appointee (Project B,C)

C. Miss Akiko Iwata, Chemist.....Technician (Project A)
Employed June 1965

D. Miss Beatrice M. Oakes, Undergraduate.....Laboratory Assistant
Employed June - Aug. 1965

E. Mr. Kenneth F. Scott, Jr., Undergraduate..... Laboratory Assistant
Employed Sept. 1965

II Status of the Grant

This original grant was designed at its infancy to undertake research which required a period of development of three to four years. However, for fiscal reasons sponsorship support was nominally decreed to extend for 21 months; yet, advice was received from the past technical monitor, Dr. Carl Bruch, that the basic plan need not be changed, and that a renewal proposal be prepared for continuation of projects for support beyond January 1966.

Thus, planning proceeded for the design and construction of a spectrophotofluorimeter which has now been completed, and installed at U.M.K.C. during the nineteenth month of the grant period. Projects B and C have shown development, but are not yet complete.

However, a renewal proposal for continued support was not successful. Permission to extend the termination date of the grant until May 31, 1966 was then requested and received, as of November 16, 1965. Some time now is available to explore instrumentally some of the intended areas of investigation of luminescence of purines and pyrimidines, first conceived in the original proposal under Project A (and, dating back to 1962). Much original thought and labor of negotiations have gone into the design of the instrument, and it is hoped that title to the instrument will ultimately reside with U.M.K.C., such that the instrument shall be available to the original investigator.

The acquisition of luminescence data in Project A, which is dependent on the instrument, is now commencing. Heretofore, activity on this project has been preoccupied with preparatory requirements, including the acquisition of purines, the recording of infrared and ultraviolet absorption spectra, the search for chromatographic systems of analysis and isolation, but especially, with the development of purification techniques which are essential to luminescence studies. What results can be achieved in this project in the next five months will be summarized in the next (final) report.

Progress on Projects B and C are included in this report, and will be summarized in the final report; however, grant support of these projects will effectively be non-existent after January, 1966.

III. Progress of Research

Project B: 1. Dye-sensitized photo-oxidation of guanine.

In the previous 8 months (9/64 - 4/65) in which the graduate assistant has been exhuming and purifying the photoproducts of this reaction it has been possible to evolve a trail of products which support the original thesis of this work: i.e. that dye-sensitized photo-oxidations, promoted by visible light can bring about chemical conversions of a representative purine and with considerably greater finesse than the chemical literature would lead one to believe. Previous reports, even of sensitized systems have given results which showed profound degradation of purine and pyrimidine ring systems; our experimental reaction control leads to products in which one carbon atom is lost from the purine.

The consequential chemistry of the primary photoproducts is the subject of Project C. The aim of Project B is the detailed study of the mechanism of the photochemical process through a study of kinetics and spectroscopic determination of substrate-dye interactions. In the second semi-annual report mention was made of instrumental difficulties. Work on the primary photochemical process has begun during the last few months.

Absorption spectrophotometry is being used to evaluate the physical state of the dye and of the dye-purine admixture at different concentration levels in order to be able to specify the species involved during changes in these concentrations during the actual excitation and photo-oxidation.

While Rabinowitch (1) and others have studied monomer-polymer association equilibria for methylene blue, these have not previously been carried out in strong alkaline solution. We have observed such association phenomena in alkaline solution, as well, but the fresh alkaline solutions, which show the monomeric absorption band at 664 mu, change very slowly to a complex absorbing at 615 mu. At a concentration level of 10^{-5} molar, methylene blue showed a rate of change of 0.1 absorbance unit per hour. The 664 mu band is completely absent in an aged solution. This change is unaffected by the presence of purine or oxygen, or the combination (in this case 8-azaguanine was used).

Some interesting characteristics of dye-substrate interaction had been observed in our photochemical process, which have now been shown to be absent among the starting materials. If the photo-oxidation is carried to a point where some guanine is still present, and only photoproduct A has been produced, this solution, if deaerated and stored in the dark will lose the blue color of methylene blue, which, however, may be regenerated by the admission of oxygen. The system acts as if photoproduct A, produced by photo-oxidation of guanine, is a reducing agent for the dye in the absence of oxygen. This supposition is suggestive of the presence of a free radical intermediate, and of a one-electron oxidation step reaction taking place.

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(1) E. Rabinowitch and L. F. Epstein, J. Amer. Chem. Soc., 63, 69 (1941); S. Granick, L. Michaelis, and M. P. Schubert, J. Amer. Chem. Soc., 62, 1802 (1940).

Project B (Con't): 2. Dye-sensitized photo-oxidation of 8-azaguanine.

8-Azaguanine represents a position labeled guanine, and, as such, the correspondence of its photoproducts, or lack thereof, to those of guanine may assist in the understanding of the above system. Because of the spectral range overlap of absorption bands in the ultraviolet region between the substrate purine and the sensitizer dye it has proved difficult to instrument for direct observation of the concentration of the substrate as a function of time in the case of guanine.

On the other hand, 8-azaguanine presents some advantages in this regard since it produces a product with a broad, long wave-length absorption band at 350-370 mμ, and, in addition, the product fluoresces strongly. Either, or both of these properties may be exploited to follow the course of product appearance. The dye concentration may easily be observed in the visible region at 622 or 670 mμ.

However, the purine substrates are still not observable directly, through some spectral window. A study is presently being carried out in which the feasibility of using the very weak fluorescence of guanine in alkaline solution at 340 mμ may be metered, quantitatively, in the reaction system.

Absorptometry of 8-azaguanine-methylene blue mixtures give no sign of complex formation in alkaline solution at 10^{-5} molar concentrations. The rate of photo-oxidation of 8-azaguanine is more sensitive than that of guanine to pH in the range 12-14, and 8-azaguanine provides considerably faster rates at high pH than does guanine.

A reaction between methylene blue and system components, on storage following an initial period of irradiation has also been observed with 8-azaguanine. Finally, in the absence of purines, methylene blue will undergo a photobleaching, which will have to be studied in alkaline solution in order to provide a correction for our primary kinetic data.

Thus, much preliminary legwork is required in our first kinetic studies of the photo-oxidation of purines.

Project C: Identification of uncommon degradation products derived from the dye-sensitized visible-light photolysis of purines.

1. Products derived from the photo-oxidation of guanine.

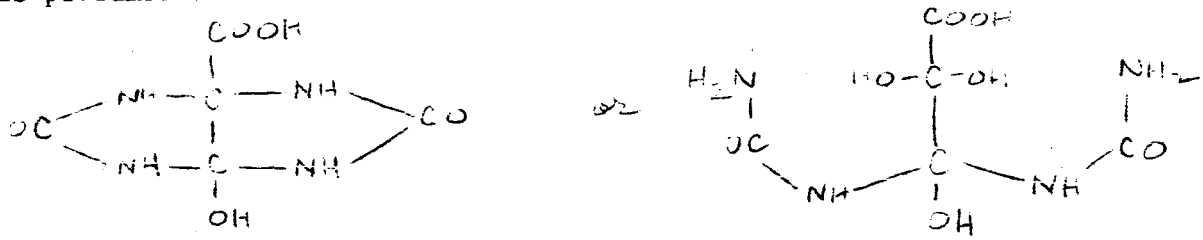
Until very recently, most of our efforts have been concentrated on a rapid identification of the products derived from the methylene blue-sensitized photo-oxidation of guanine in alkaline solution using red light. It is now evident that structural identification shall not be rapidly accomplished.

The two previous semi-annual reports described the isolation of a product B, formed photolytically from the primary photoproduct, A, and products C and D, formed by acid treatment of the primary photoproduct. The reports also describe our determination of molecular weights and titration results for products B and C. The products, however, while chromatographically homogeneous, have challenged the abilities of three commercial analytical laboratories, who have carried out elemental analyses on check samples with contradictory results, by inter and intra-

comparison. The establishment of reputable elemental analysis had been set as a critical goal; our course, now, however, is to degrade the products further to recognizable derivatives.

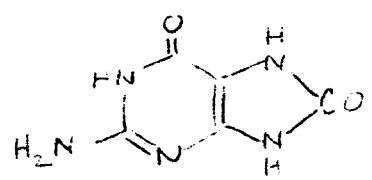
The analyses, however, do establish the fact that the carbon:nitrogen: hydrogen:oxygen content for these products is such that structures are probably relevant to the necessary and interesting, but still unknown degradation pathway of conversion of uric acid to allantoin, alloxan, and parabanic acid end products. The degradation of uric acid by chemical (2),enzymatic (3),and electrolytic (4) means has been studied and evidence tends to indicate that a uniformly common mechanism does not exist for these degradative oxidations of uric acid. Dye-sensitized oxidation would represent a fourth alternative, but this has not been determined. Lumichrome-sensitized ultraviolet photo-oxidation of guanine has yielded parabanic acid (1); our dye-sensitized photo-oxidations has stopped the degradation at a much earlier stage, since (C_4N_5)- and (C_4N_4)-products are isolated, and parabanic acid is not produced.

Uric acid has been known for 190 years, but the chemistry of its oxidation route is still undetermined. Speculation in the last 15 years of a key intermediate has centered on the "Behrend Compound", the structure of which (again, speculative) is presumed to be:

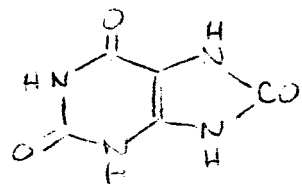


Struck and Elving (4) believe they have evidence of another key intermediate in addition to and a precursor for the Behrend Compound, in their electrolytic oxidations.

Since, on the basis of electron densities and the chemistry of purines, oxidation in the 8-position is a reasonably probably prospect, one might assume that 2-amino-6,8-dihydroxypurine (I) is a likely derivative from the oxidation of guanine in the early stages. As such,it represents an amino analog of uric acid.



(I)



URIC ACID

(2) H. Brandenberger, Experentia, 12, 208 (1956).

(3) G. Soberon and P. P. Cohen, Arch. Biochem. Biophys., 103, 331 (1963).

(4) W. A. Struck and P. J. Elving, Biochemistry, 4, 1343 (1965).

(5) J. S. Sussenbach & W. Berends, Biochem. Biophys. Acta, 16, 263 (1964); also private communication, 12/15/64.

(6) M. I. Simon and H. VanVunakis, 105, 197 (1964).

A consideration of mechanisms for the dye-sensitized degradation of guanine results of Berends (5) on the one hand, and the dye-sensitized degradation of theophylline results of Simon and Van Vunakis (6) on the other, leads this director to believe a symmetrical bi-ring intermediate, such as the Behrend Compound, is a key intermediate in these photo-oxidation of purines.

On the basis of elemental analysis, our isolated products are at the level of oxidation of the Behrend Compound. Following structural proof of our photoproducts, should they relate to the intermediates in uric acid oxidation, we will have provided access to compounds for study, and contributed to the eventual solution of the uric acid mystery.

2. Products derived from the photo-oxidation of 8-azaguanine.

Because the 8-position in 8-azaguanine is occupied by nitrogen, successful photo-oxidation of this compound and comparison of its kinetics and chemical structure of its products with the results of similar treatment of guanine may well bear on the question of the degree of reactivity of the 8-position in guanine, relative to some other position for oxidative attack in alkaline solution.

The methylene blue-sensitized photo-oxidation of 8-azaguanine in alkaline solution was studied for three months during the summer, with the following results:

a. 8-Azaguanine undergoes dye-sensitized photo-oxidation, and does so more readily than guanine.

b. One major product derivative is indicated by thin layer chromatography, and may be isolated by acetone addition to the reaction mixture, and then further purification.

c. It is an acid with a pK_a of 3.9, but is hydrolyzed in solution at pH 2 at room temperature.

d. It has an ultraviolet absorption spectrum very similar to 2,4,6-trihydroxypteridine, with band maxima at 218, 257, and 350-370 mμ (broad) of relative intensities, respectively, 1:0.8:0.1.

e. The product is fluorescent, another similarity with pteridines.

Hydrolytic degradation of uric acid has been shown to yield pteridines (7,8), and products of higher degree of polymerization. (The latter fact heightens our interest in the structure of photoproduct B which we obtain in the photo-oxidation of guanine, and have tentatively shown to be tetrameric.) Studies of the general metabolism of 8-azaguanine (9) have also yielded highly fluorescent products of undetermined structure. The value of our efforts of isolation, identification of structure, and characterization of the derivatives of purines from our productive photosynthetic method is obvious, since a large background of identified purine derivatives is lacking, and dye-sensitized photosynthesis can provide a ready access to some of these compounds.

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(7) F. G. Hopkins, Proc. Roy. Soc.(B), 130, 359 (1941-2).

(8) W. Pfleiderer, in Ciba Foundation Symposium Conference on Purines, Little Brown & Co., Boston, 1957, p. 84.

(9) R.E.F. Matthews, ibid, p. 277

IV. Other Activities

A. Lectures presented.

1) Dr. Millich presented a paper at the 150-th national meeting of the American Chemical Society, September 14, at Atlantic City, New Jersey, entitled, "The Chemistry and Polymerization of a New Class of Polymers: Polyisocyanides".

2) Dr. Millich presented a paper at the First A.C.S. Midwest Chemistry Conference, November 4, at Kansas City, Missouri, entitled, "Chemical Products from Methylene Blue-sensitized Photo-oxidation of Guanine". (F. Millich and H. Nanaie).

3) Dr. Millich had a paper presented at the First A.C.S. Midwest Chemistry Conference, November 4, Kansas City, Missouri, entitled: "Characterization of Poly(α-phenylethylisocyanide)", (F. Millich and R. G. Sinclair).

B. Other meetings attended.

1) Dr. Millich attended a two week chemistry conference on Quantum Chemistry, July 26 - August 6, at the University of Wyoming, Laramie.